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Reduction of molecular gas diffusion through gaskets in leaf gas exchange cuvettes by leaf-mediated pores

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ABSTRACT

There is an ongoing debate on how to correct leaf gas exchange measurements for the unavoidable diffusion leakage that occurs when measurements are done in non-ambient CO₂ concentrations. In this study, we present a theory on how the CO₂ diffusion gradient over the gasket is affected by leaf-mediated pores (LMP) and how LMP reduce diffusive exchange across the gaskets. Recent discussions have so far neglected the processes in the quasi-laminar boundary layer around the gasket. Counter intuitively, LMP reduce the leakage through gaskets, which can be explained by assuming that the boundary layer at the exterior of the cuvette is enriched with air from the inside of the cuvette. The effect can thus be reduced by reducing the boundary layer thickness. The theory clarifies conflicting results from earlier studies. We developed leaf adaptor frames that eliminate LMP during measurements on delicate plant material such as grass leaves with circular cross section, and the effectiveness is shown with respiration measurements on a harp of *Deschampsia flexuosa* leaves. We conclude that the best solution for measurements with portable photosynthesis systems is to avoid LMP rather than trying to correct for the effects.

Key-words: CLIMAITE project; CO₂ leakage; diffusion leakage; gasket density; leaf adaptor frame (LAF); leaf respiration; portable gas exchange system.

INTRODUCTION

Small leaf chambers are widely used for measurements of leaf gas exchange. Measurements of small gas fluxes such as leaf respiration strongly depend on accuracy, and even small artificial changes of the CO₂ flux can be of significant magnitude relative to the correct rate of leaf gas exchange (e.g. Bruhn, Mikkelsen & Atkin 2002; Pons *et al.* 2009). Unfortunately, diffusion through the gasket material in modern commercial portable leaf gas exchange systems is unavoidable and has been demonstrated and described previously (e.g. Long & Bernacchi 2003; Flexas *et al.* 2007; Rodeghiero, Niinemets & Cescatti 2007). Most manufacturers provide methods to correct for the diffusion, and different methods to avoid or minimize the advective leakage through gaps between plant and gasket material, that is, leaf-mediated pores (LMP), and have been suggested. Rodeghiero *et al.*

(2007) suggested enclosing the leaf chamber in a bag and let the gas concentration inside the bag approach that inside the leaf chamber. Flexas *et al.* (2007) observed that using dead or inactive broadleaf material as reference for correction resulted in more reliable flux estimates than using the manufacturer's correction method alone. However, these methods are difficult, if not impossible, to apply under extensive field work, and correction with specific dead leaf material is not useful concerning small leaf structures such as grasses, where the number of leaves and thereby LMP between the gaskets cannot be kept constant. Whereas Flexas *et al.* (2007) only describe the effects of using dead leaf material, this study aims at suggesting a theory that can be used to understand and correct the unintended effects of LMP in gas exchange equipment. We tested the theory using the portable photosynthesis system LI6400 (Li-Cor Inc., Lincoln, NE, USA). A newly developed leaf adaptor frame (LAF) for measurements of small but thick leaves, that are of much smaller width than the cuvette opening area, is tested for minimizing the effects of LMP. The use of LAF aims to measure reliable gas exchange rates under field conditions, even at small fluxes such as leaf dark respiration, and to conduct repeatable and reproducible *in situ* measurements on exactly the same plant material in a sequence of measurements.

Further, we investigate the effects of different diffusion correction methods applied to field measurements on Wavy hair-grass (*Deschampsia flexuosa*). Finally, we aim at recommending a procedure that minimizes errors in leaf respiration measurements.

THEORY

The development of LAF aimed at minimizing possible leakage effects through LMP. However, pilot studies of the relation between using LAF and the effects of artificial LMP showed that the opposite was the case: Diffusive losses in gas exchange measurements were lower in presence of LMP than without. The following theory describes the influence of LMP on the accuracy and precision of leaf gas exchange measurements, and is able to answer the obvious question: 'How can LMP reduce leakage through the gaskets of gas exchange cuvettes?'

Direct leakage, caused by leaf structures creating small pores between the gaskets, will result in a mass flow of air between the chamber and the surrounding air. To avoid an inflow of air into the chamber, a small overpressure inside the

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chamber is maintained. In the situation of a completely sealed chamber, that is, no pores that allow advection of air between the inside and the outside of the chamber, gasses will diffuse through the gaskets according to the concentration gradient across the gasket and the diffusion coefficient of the sealing material. This situation is the basis for the manufacturers' correction (Li-Cor Inc. 2008).

Gas diffusion through a material can ideally be described by Fick's first law,

$$F_D = -\frac{K_D(\rho_o - \rho_i)}{S_g l}, \quad (1)$$

where F_D is the flux across the material due to diffusion out of the cuvette ($\text{mol m}^{-2} \text{s}^{-1}$), $\rho_o - \rho_i$ is the difference in molar density between the inside (index i) and the outer surface (index o) of the material (mol m^{-3}), S_g and l are the area and length of the path through the compressed gasket (m), respectively, and K_D is the diffusion coefficient of the compressed gasket material ($\text{m}^2 \text{s}^{-1}$) for the gas of interest. The term $\frac{\rho_o - \rho_i}{l}$ can be referred to as the concentration gradient.

In a completely closed chamber, that is, without any open pore between the gaskets, the total loss or gain of CO_2 according to the measurement can be described with Eqn 1, where the diffusion leakage only varies with the CO_2 concentration gradient.

In a situation where leaves or other plant organs create small pores as a result of their structure, the overpressure inside the chamber results in a continuous loss of air from the inside of the cuvette caused by mass flow through the pores. Such advective loss of air (and thereby a given gas mass transport) from the interior of the chamber can be described as,

$$F_{\text{air}} = v C_i, \quad (2)$$

where v is the volumetric flow of air ($\text{m}^3 \text{s}^{-1}$) and C_i is the concentration of CO_2 (mol m^{-3}) inside the chamber (Fig. 1). Because of the small overpressure in the chamber, F_{air} is outward directed. Therefore, it is assumed that F_{air} does not affect C_i and thus the flux measurements. It will, however, be shown that this is an oversimplification.

The total flux of CO_2 across the gasket in the presence of LMP, that is, $F_t = F_D + F_{\text{air}}$, is depending on the direction and the magnitude of the CO_2 concentration gradient across the gasket (Fig. 1). Here, it is important to consider the CO_2 concentration in the boundary layer surrounding the gasket and the wind conditions surrounding the leaf cuvette. In Figure 2, two possible scenarios are shown, where the CO_2 concentration inside the chamber is higher than the ambient CO_2 concentration outside the cuvette. With increased thickness of the boundary layer, the CO_2 concentration in the quasi-laminar boundary layer (C_{btl}) will be charged with gas from the inside as a result of the advective mass flow, and thus, C_{btl} approaches C_i , a situation that normally would occur under indoor conditions (Fig. 2a). As a consequence, the concentration gradient decreases, and F_t decreases as a result of decreased F_D through the gasket

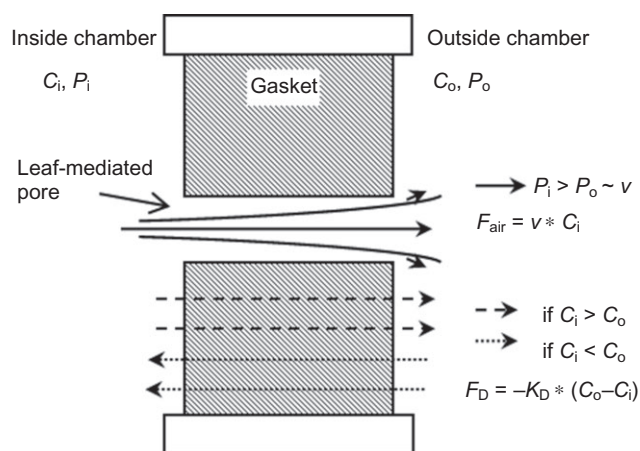


Figure 1. Schematic presentation of the mass flow (black arrow) and diffusion (dotted, dashed arrow) in different concentration situations where leaf-mediated pores are present. Sketch dimensions are not real to improve the illustration of the theory. F_{air} is the rate of advective CO_2 mass flow out of the chamber depending on the volumetric flow of air (v) and the CO_2 concentration inside the chamber (C_i). P_i and P_o are the pressure inside and outside of the chamber, respectively. F_D is the rate of CO_2 loss/increase as a result of diffusion depending on the diffusion coefficient K_D and the difference in CO_2 concentration outside and inside the chamber ($C_o - C_i$).

material. The thickness of the boundary layer decreases with increasing wind velocity at the surface (e.g. Nobel 1991). Thus, under windy conditions that are typical for outdoor measurements, a higher CO_2 concentration gradient will be kept across the gasket, thus increasing F_D (Fig. 2b). In non-windy conditions, the effect of LMP will thus, counter-intuitively, lead to a smaller diffusive flux across the gasket as compared to a gasket without plant-mediated pores. The effect of LMP on the diffusion through the gasket will be as variable as are the wind conditions in the field.

With no LMP present, the windy conditions around the leaf cuvette should, according to the theory, reduce the concentration gradient, but because of the lack of additional mass flow of air from the inside of the cuvette, this effect would be much smaller if not insignificant. To support the theory, we tested the following three hypotheses: (H1) the leakage flux is a problem of diffusion through the gasket, (H2) LMP reduce the diffusive flux in non-windy conditions and (H3) in windy conditions, the reducing effect of LMP on the diffusion leak is approaching the diffusion without LMP. H1 was tested by sealing the cuvette completely with a gas-tight material and investigate the leakage at a strong CO_2 concentration gradient. If replacing the gasket by gas-tight material removes the leakage, the leak observed when using gaskets must be caused by molecular diffusion through the gasket material. H2 was tested by comparison of empty cuvette measurements with gaskets and measurements with LMP caused by leaf artefacts. H3 was tested with the same approach as for H2 at differing wind speeds outside the gasket.

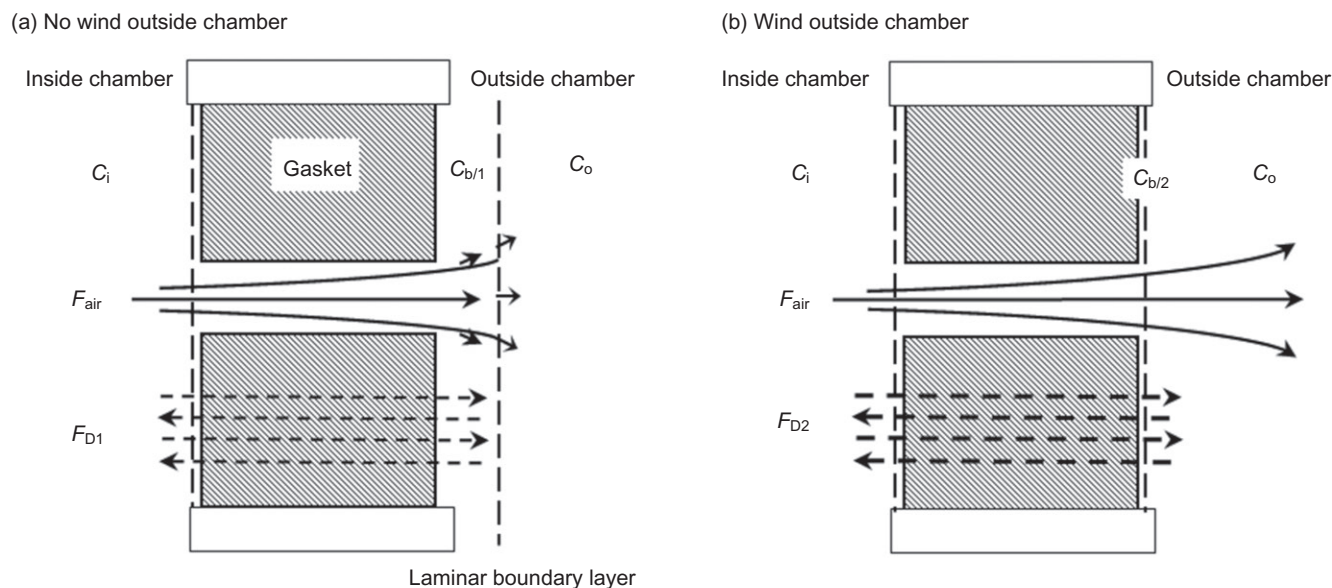


Figure 2. Schematic presentation of the diffusion theory under two turbulent regimes on the outer side of the chamber gaskets. F_{air} is the rate of advective mass flow of CO_2 depending on the velocity of the mass flow of air (v) and the CO_2 concentration inside the chamber (C_i). F_{D1} and F_{D2} are the rates of CO_2 transport as a result of diffusion depending on the diffusion coefficient K_D and the CO_2 concentration gradient across the gaskets, in the two cases (a and b). C_{b1} and C_{b2} are the concentrations of CO_2 in the boundary layer directly at the outer surface of the gasket in the two cases (a and b) and C_o is the concentration in the surrounding air outside the chamber.

MATERIALS AND METHODS

Plant material and locations

Wavy hair-grass (*Deschampsia flexuosa* (L.) Trin.) was used for the experiments with living plant material. We used this plant because accurate gas exchange field measurements on *D. flexuosa* and *Calluna vulgaris* were needed for the multifactorial climate manipulation experiment, CLIMAITE, Brandbjerg, North-Zealand, Denmark (see Mikkelsen *et al.* 2008). With respect to gas exchange measurements, the plant species has the disadvantage of having small and thick leaves. For gas exchange measurements, a bundle of 10–15 parallel leaves were fixed carefully inside an aluminium frame (LAF, see below), avoiding overlapping leaves, that is, a similar procedure as in Albert *et al.* (2011a).

Regular leakage tests with empty cuvettes were conducted about every second week during 2011 in the experimental area of CLIMAITE. Outdoor measurements were all done under the specific environmental conditions at ambient CO_2 concentrations. Indoor measurements were conducted at two different places with differing background CO_2 concentrations. Analyses with gaskets were performed in a well-ventilated room at ambient CO_2 concentrations around 400 ppm. All other tests were conducted under controlled CO_2 concentrations in a fume cupboard with continuous ventilation. The CO_2 concentration in the cupboard was continually measured and logged with a LI-7550 infrared gas analyser (Li-Cor) connected to a laptop computer. The background CO_2 concentration in the cupboard was 454.5 ± 0.3 ppm ($n = 12\,346$) during all measurements.

LAF description

The LAF consisted of two small aluminium frames 40×60 mm (1 mm sheet). Each frame had an opening matching exactly the dimensions of the cuvette opening of 20×30 mm. The LAF was infolded with a 4 mm fold (Fig. 3a,b). The two frames and the plant material were sealed with blue tack (Lyreco, Marly, France), that is, establishing similar surface contact conditions to the gaskets as large flat leaves. The blue tack was proven to be gas tight and thereby suitable for sealing the LAF (presented under ‘Diffusion tests with and without LAFs’). In each of the folds, there were eight holes (\varnothing 1 mm) to establish a harp of 0.3 mm nylon strings, in order to support *C. vulgaris* shoots inside LAF and guarantee minimal shoot overlap. The usefulness of this feature is not tested here.

Gas exchange measurements

The present study is performed using the LI-6400 open Portable Photosynthesis System from Li-Cor Biosciences, a type of system widely used for leaf level gas exchange measurements. The LI-6400 was connected to a standard 20×30 mm chamber with a LED light source (6400-02B) and a CO_2 -mixing device controlling the level of reference CO_2 . Other manufactures provide similar systems, and the theory applies for all portable photosynthesis systems that provide a slight overpressure inside the chamber and are sealed with non-gas-tight foam material.

We used the following protocols for CO_2 response curves and light response curves in the field. Leaves were

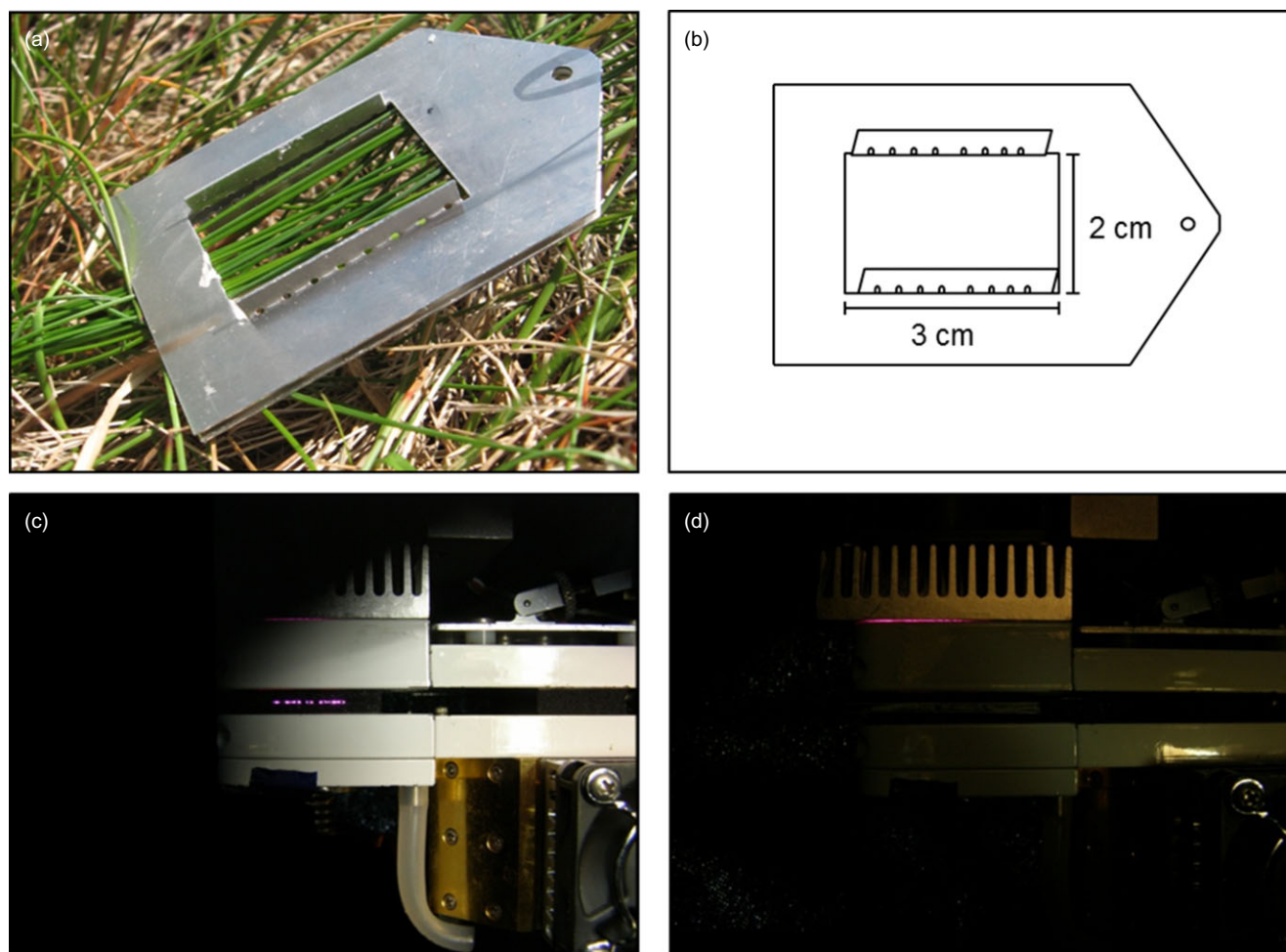


Figure 3. (a) The use of leaf adaptor frames (LAF) at the field site. *Deschampsia flexuosa* leaves are attached in LAF and ready for measurements. (b) Schematic sketch of LAF, in correct scale. (c) Visual illustration of the occurrence of artificial leaves (AL). The purple light comes from the RBG (red-blue-green) light source of LI6400. No light can be seen when the AL are sealed with blue tack in the LAF. (d) The same as (c) but here, the AL are kept inside LAF.

acclimated to the chamber condition for 6 min at 390 ppm, until net photosynthesis and stomatal conductance were stabilized [coefficient of variation (CV) < 1%]. A CO₂ response curve was measured stepping down the CO₂ concentration from 390 to 50 ppm CO₂ and then re-establishing it to the 390 ppm level again, for at least 3 min. Then, the concentration of CO₂ was stepped up to complete saturation at 1400 ppm CO₂. Measurements were performed at a light saturating level of 1500 μmol photosynthetically active photons $\text{m}^{-2} \text{s}^{-1}$, using the Li-6400 auto-program 'ACi-curves' with these settings: time between measurements min 45 and max 55 s, reference CO₂ (C_i) [mol mol^{-1}] and intracellular CO₂ concentration (c_i) stable in 10 s with CV < 1%. Matching was performed between every step. Block temperature was set to 25 °C. Relative humidity was adjusted to 45–60% during measurements. Non-photochemical respiration (R_{light}), maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates were calculated from curve fitting to the Farquhar–von Caemmerer–Berry (FvCB) model equations

(Bernacchi *et al.* 2001; Dubois *et al.* 2007). Immediately after running the ACi-curve protocol, the light response curve was measured. The auto-program 'Light curves' on the Li-6400 was used by stepping down the light from 2000 μmol photosynthetic photons $\text{m}^{-2} \text{s}^{-1}$ [photosynthetic active radiation (PAR)] in nine steps to zero. The photosynthesis saturating reference CO₂ concentration was set to 1400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. From the light response curve the maximum dark respiration (R_{dark}) and maximum light-saturated rate of photosynthesis (A_{max}) was calculated using a non-rectangular hyperbola as regression model (Lambers, Chapin & Pons 1998). In a last step, leaf dark respiration (R_D) was measured directly in the dark at 390 ppm, that is, ambient CO₂ concentration, and estimated from 6 min of flux data at 2 s resolution.

All data were recalculated for correct leaf area and corrected for leakage with three different methods (see further details in the paragraph 'Data corrections').

The three different estimates for leaf respiration (R_{light} , R_{dark} , R_D) are compared.

Diffusion tests with and without LAFs

Tests were conducted to evaluate the influence of the use of a chamber gasket for sealing (H1). Firstly, the leaf chamber was completely sealed with blue tack not using gaskets at all. The differences of the fluxes in a completely sealed versus a gasket-sealed empty cuvette are thus caused by diffusion through the gasket. We assumed that the fluxes measured with a completely sealed empty cuvette are zero $\mu\text{mol CO}_2 \text{ s}^{-1}$.

The second test was done by comparing two different chamber gasket materials, white gaskets (spare part no. 6400-30 and black gaskets (spare part no. 6400-33). Three different combinations were obtained using either only white or black or a combination of the two (upper and lower).

Thirdly, the use of an empty LAF was compared to an empty chamber, in both cases sealed with gaskets.

In a fourth test, the effects of pores across the gasket, established by using a bundle of seven tin solder wires (\varnothing 0.8 mm) that mimic the dimensions of grass leaves (henceforth artificial leaves, AL), was investigated either with or without using LAF (H2).

Effects of ALs on the pressure difference between inside and outside the cuvette

To test whether or not there is a pressure difference across the gaskets at different regimes, a needle attached to a pressure sensor (Model 278, Setra System Inc., Boxborough, MA, USA) was inserted through the gasket.

Effects of wind on gas exchange measurements

To evaluate the effect of turbulence around the leaf cuvette (H3), tests with AL with or without LAF in otherwise empty chambers were conducted under two conditions: with a small fan (model: embpapst 412 (Embapast, Brøndby, Denmark) with an approximate wind speed of 0.35 m s^{-1} close to the fan) in front of the chamber, creating turbulence in the air. This was compared to the same measurements in still air, also taking care that the Peltier cooler would not generate a wind field under those measurements.

Area estimations

After the measurements were performed, the LAF containing the leaves were cut off. The LAF with leaves were placed in a flatbed scanner with the light-exposed side downward and scanned. To avoid damaging the scanner, LAFs were placed inside a gasket attached to a transparency sheet. The non-light-exposed side was filled with gasket material to avoid shading effects. Area estimations were quantified using the image processing program (ImageJ, National Institute of Health, Bethesda, MA, USA). The inside length of the LAF (3 cm) was in all cases the reference length, and area was determined from 8-bit colour pictures with the threshold approach. The scanned leaf areas are given as projected leaf areas, after Smith, Schoettle & Cui (1991).

Data correction

The correction for even small leaks is important for the correct estimation of leaf respiration, because it is itself a relatively small flux. Two different methods to correct for diffusion through the gasket are used and compared.

Firstly, the manufacturer provides a flow-dependent normalized diffusion rate $k = 0.46 \text{ (mol s}^{-1}\text{)}$. The CO_2 gas exchange rate can be corrected using the following equation:

$$A_K = \frac{u(C_r - C_s)}{100S} - C_s E + \frac{k}{100S}(C_a - C_s), \quad (3)$$

Where A_K is the corrected assimilation rate, u is the flow rate through the chamber, E is the calculated transpiration rate to account for the CO_2 dilution through water vapour flux from the leaf, S is the leaf area inside the chamber (cm^2). Note that C_r , C_s and C_a are the mole fractions (mol mol^{-1}) in the reference cell, sample cell and in the surroundings, respectively, and not the molar densities as in Eqn 1). The first term of the Eqn 4) represents the assimilation rate without any diffusion correction and the last term is the correction term, $\frac{k}{100S}(C_a - C_s)$. The k -value provided by the manufacture is estimated for the use of one white and one black gasket (Li-Cor Inc. 2008). Note the differing sign conventions: Li-Cor provides assimilation rates, which are positive when the leaf takes up CO_2 via photosynthesis. In a physical gas exchange perspective, positive fluxes are a result of CO_2 addition to the chamber, which in biology refers to the situation of leaf respiration.

The relationship between k , u , C_r and C_s can be expressed as

$$\frac{C_s - C_r}{C_r} = -\frac{k}{u}. \quad (4)$$

This relationship is used for estimation of k for the use of two black gaskets and the use of LAF with AL. These different k -values will later be used for correction of photosynthesis rates.

An alternative empirical method to correct the data is using an empty chamber approach, described by, for example, Bernacchi *et al.* (2001). All C_s (sample cell) values on the ACi curves are corrected with the corresponding $\Delta C_E = C_{s,E} - C_{r,E}$, from a LI6400 machine-dependent mean of empty chamber measurements for each of the concentration levels. The mean ΔC_E used in this study is based on a minimum of 35 machine-dependent, empty chamber measurements done in the field across the season of 2011. After adding the correct area, the corrected assimilation rate (A_E) is calculated as:

$$A_E = \frac{u(C_r - (C_r - \Delta C_E))}{100 * S} - (C_s - \Delta C_E) * E, \quad (5)$$

where A_E is the empty-apparent assimilation rate measured in an empty cuvette and u is the flow rate through the chamber. This approach assumes that the atmospheric concentration is invariant, which is justified because the measurements were taken at daytime when the atmosphere is well

mixed. The seasonal variation of the ambient daytime CO₂ concentrations is small compared to the range of C_i and the ACi and light response protocol.

Statistical analysis

Statistical analyses are done using the R software (R Development Core Team 2010). The linear dependency of F_D to the concentration gradient across the gasket was tested with linear regression. We represented F_D by the measured concentration difference ($C_s - C_i$), which is proportional to the flux because the flow rate of air through the cuvette under the experiment was held constant. The concentration gradient was represented by the CO₂ concentrations in the cuvette, noting that the ambient concentration was held constant under the experiment. The reason for doing so is firstly, that these values are given as data pairs from the analyser, and further, as the results will show, that the relevant C_a , that is, the concentration directly at the outer surface of the gaskets was not possible to measure, anyway. The advantage is that the results can directly be compared (all concentration units) and interpreted. The slopes of ($C_s - C_i$) versus C_i were tested to be different from zero. If the slope differed from zero, this was a result of diffusion. Differences between slopes of different experiments were tested using pairwise *t*-test and Tukey's grouping test. The differences between different correction methods and the three different respiration estimates were also tested for significance using pairwise *t*-test and Tukey's grouping test.

RESULTS

Effects of LAFs on diffusive leakage

Measurements with completely blue tack-sealed cuvettes did not show any linear relationship between the flux, represented by the concentration difference in the air before entering and after leaving the cuvette ($C_s - C_i$), and internal CO₂ concentration inside the cuvette (C_i ; Fig. 4). C_i in this case represents the concentration difference across the blue tack material because the outside concentration was held constant running the CO₂ response protocol ($P = 0.88$, $R^2 = 0.012$). The same result was found when testing the effect of eventually remaining artificial pores introduced in the blue tack sealing ($P = 0.37$, $R^2 = 0.00$). The effective sealing of the LMP was also demonstrated visually comparing Fig. 3c and d (see below). The non-linear patterns that both treatments showed in Fig. 4 are not significant. They can be seen in all test measurements and are machine dependent (see, e.g. Fig. 4). These interesting patterns are not the subject of this study but we note that there is a small, systematic under or overestimation of the flux beyond the leakage through the gasket in this particular cuvette design depending on the choice of C_i .

Sealing the leaf chamber with gaskets showed a linear relation of the artificial CO₂ flux with the CO₂ concentration inside the chamber ($P < 0.001$, $R^2 > 0.5$; Fig. 5). There was a significant difference between the different gasket materials

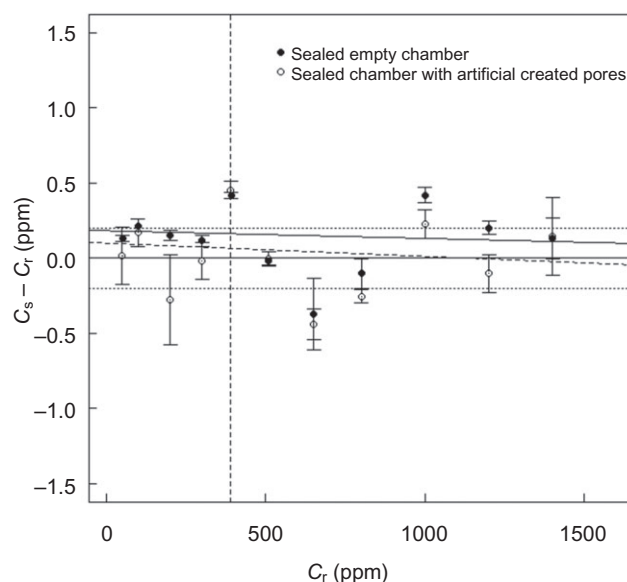


Figure 4. CO₂ concentration differences between the sample (C_s) and the reference cell (C_i) and the CO₂ concentrations inside the leaf chamber (C_i) for measurements done with blue tack-sealed chamber (●) and blue tack-sealed chamber with artificial created pores (○). Solid and dashed lines are the linear regression lines for sealed and sealed with artificial created pores, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO₂ concentration in the surroundings during measurements.

($P = 0.04$). If two white gaskets were used, the regression lines were significantly different from the regression using two black gaskets ($P = 0.018$). Figure 5 shows that the diffusion was highest using the white gaskets. The combination of a black and white gasket resulted, as expected, in intermediary diffusion rates, with significantly different slopes compared to the use of white gaskets ($P = 0.018$). Table 1 shows the estimated k -values for all cases. The k -value from white/black gasket accurately confirmed the Li-Cor k -value of 0.46 mol s^{-1} .

Testing the effect of an empty LAF compared to an empty cuvette did not show any significant difference between the slopes ($P = 0.24$). In both cases, there was a clear linear relationship with increasing CO₂ concentration inside the leaf chamber; both slopes were significantly different from zero ($P < 0.001$, $R^2 > 0.35$).

We used a qualitative test to show the existence of LMP, by illuminating the cuvette from the inside with the instrument internal red light source. In Fig. 3c, LMP caused by AL are visualized by the light shining between them towards the outside of the cuvette. When sealing the AL with blue tack inside the LAF, no light could be seen from outside (Fig. 3d). Comparison of the linear relationship between the fluxes and the CO₂ gradient using AL kept between the two black gaskets and AL kept in the LAF, showed a significant difference between slopes ($P < 0.001$). The slope was smaller for AL alone than AL fitted inside LAF; however, both were different from zero ($P < 0.001$, $R^2 > 0.6$). The k -value

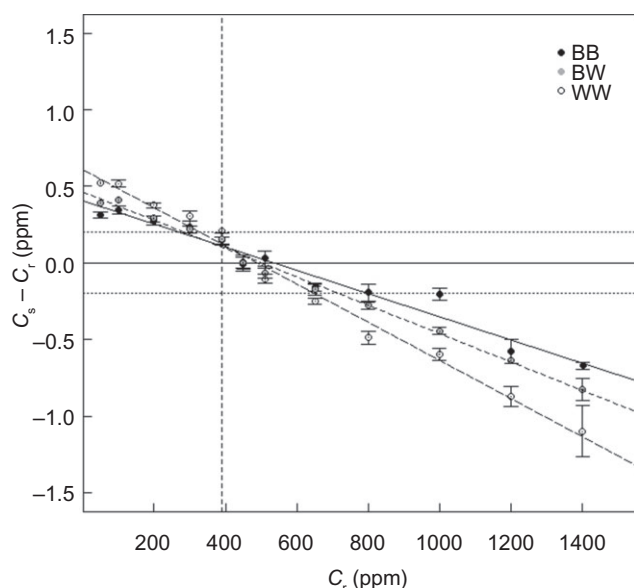


Figure 5. Influence of sealing an empty chamber with different gasket combinations, black/black (●), white/black (grey ●) and white/white (○), on the CO_2 concentration difference between the sample (C_s) and reference air in relation to the inlet CO_2 concentration (C_r). Solid, dashed and dotted lines are the linear regression lines for measurements with black/black, white/black and white/white gaskets, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO_2 concentration in the surrounding air during measurements (approximate 400 ppm CO_2).

using two black gaskets were $k_{\text{BB}} = 0.38 \pm 0.02 \text{ mol s}^{-1}$ and $k_{\text{LAF}} = 0.38 \pm 0.025 \text{ mol s}^{-1}$ when AL were kept inside LAF (Table 1).

There was a detectable but very small increase of the pressure inside of the AL chamber compared to outside (103.57 kPa outside to 103.59 kPa inside). Artificial pores created with needles ($\varnothing = 0.8 \text{ mm}$, i.e. much larger than the LMP caused by AL) did not change this pressure difference.

Disturbance of the boundary layer surrounding the gasket with a fan reduced the concentration gradient. However, it was not significant when AL were kept inside LAF ($P = 0.86$). Wind had a significant effect when AL were kept between gaskets ($P < 0.01$), that is, LMP were established between the gasket and AL.

Effect of the correction method on the respiration estimates

Photosynthetic model parameters based on CO_2 - and light response data from four individual plants of *D. flexuosa* were corrected using two different methods, correction with the Li-Cor provided correction term using two different diffusion rates, $k = 0.46$ and $k_{\text{BB}} = k_{\text{LAF}} = 0.38$, and using the mean of 35 empty chamber measurements collected throughout the year 2011 under many different kinds of environmental conditions (wind: c. 0–15 m s^{-1} , temperature: c. 0–30 °C and humidity: c. 50–99%; $P < 0.001$, $R^2 = 0.35$).

The only parameters of the FvCB model (A/C_i) that were significantly affected by the correction method were the respiration parameters (Table 2). Both correction methods, that is, the k -value approach or subtraction of empty cuvette measurements from the measurements, resulted in a significantly lower respiration rate compared to the non-corrected respiration rate ($P < 0.008$). In addition, the result of the light response fitting only showed a significant influence of the correction method in the parameterization of respiration (Table 2). No difference was found between the two correction methods. Dark respiration measurements were not affected by any correction, which was expected as they were performed under ambient concentrations and serve as a reference.

The three different respiration estimates (R_{light} , R_{dark} and R_D) were significantly different from each other when no correction of the data was done ($P = 0.001$). In contrast, no difference was found when data were corrected by either of the k -values ($P > 0.35$) or ΔC_E ($P = 0.048$). The only parameter where the correction method influenced the result was R_{light} . R_{light} corrected with $k_{\text{BB}} = 0.38$ was significantly different from the ΔC_E -corrected R_{light} ($P = 0.015$). When R_{light} was corrected by k , it tended to be different from the ΔC_E -corrected R_{light} . No difference was seen between k and k_{BB} corrected parameters.

DISCUSSION

The influence of LMP on gas exchange measurements

Above all, to note is that the CO_2 leakage from or into the leaf chamber is a result of diffusion determined by the gasket material. Sealing the leaf chamber with gas-tight material stopped any diffusion, as proven by the measurements, that is, the absence of any relationship between the measured flux in the empty chamber and the CO_2 concentration inside the leaf chamber. Even when LMP were artificially introduced through the gas-tight sealing material, no effects of the concentration gradient were seen (Fig. 4). This supports the initial hypothesis of the manufacturer that a

Table 1. Estimates of diffusion coefficients (k) with different gaskets combinations, with or without AL or inside LAF

Treatment	Intercept	Slope	R^2	k -value
WB	0.47	−0.00092	0.98	0.46
WW	0.61	−0.00124	0.96	0.62
BB	0.41	−0.00076	0.93	0.38
LAF + AL	0.40	−0.00077	0.89	0.38
AL	0.33	−0.00064	0.82	0.32

All k -values are calculated using a flow rate (u) at 500 mol s^{-1} under indoor conditions in a well-ventilated room.

WB, one white and one black gasket; WW, two white gaskets; BB, two black gaskets; LAF + AL, artificial leaves attached between two black gaskets using LAF; AL, artificial leaves attached between two black gaskets without using LAF; R^2 , adjusted R -squared.

Parameter	No correction	$k = 0.46$	$k_{BB} = 0.38$	ΔC_E
FvBC model				
V_{cmax}	183.83 \pm 32.0	167.6 \pm 29.6	170.1 \pm 29.6	166.4 \pm 31.1
J_{max}	212.9 \pm 27.2	194.6 \pm 27.4	197.8 \pm 27.4	189.6 \pm 27.8
R_{light}	7.8 \pm 0.2	5.7 \pm 0.1***	6.0 \pm 0.1***	5.0 \pm 0.3***
Light response model				
A_{max}	63.3 \pm 11.3	63.2 \pm 11.3	63.2 \pm 11.3	63.1 \pm 11.3
R_{dark}	2.9 \pm 0.3	5.9 \pm 0.7*	5.4 \pm 0.7†	5.7 \pm 0.7*
Dark respiration				
R_D	5.1 \pm 0.1	5.1 \pm 0.1	5.1 \pm 0.1	5.0 \pm 0.1

Table 2. Comparison of different correction methods for the estimation of physiological parameters from raw gas exchange data

All data are collected using LAF. Data are corrected by the Li-Cor diffusion coefficient $k = 0.46$, with the estimated $k_{BB} = 0.37$ for black gasket or by ΔC_E mean of outdoor empty chamber measurements ($n > 35$). R_{light} is the respiration in light extracted from the FvBC model from A/Ci curves, R_{dark} is the dark respiration extracted from the light response model and R_D is the actual measured rate of respiration under ambient conditions. V_{cmax} is the maximum carboxylation rate, J_{max} the maximum capacity of electron transport and A_{max} is the maximum light and CO₂ saturated photosynthesis. Significance levels are given as † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In no cases, the three corrected values were significantly different from each other.

small overpressure in the cuvette will offset any effects of LMP on the cuvette internal concentrations. The small slope seen in Fig. 4 with LMP present is due to a mass loss of CO₂ across the cuvette gaskets, but it is not significant. In Fig. 5, the results of testing different gasket materials and their combination show significant linear relationships between the flux and the CO₂ concentration inside the leaf chamber, representing the concentration gradient across the gaskets as the external concentration was held constant. These slopes differed significantly using different gasket material. In the light of the presented theory on diffusion and the fact that mass flow is only outward directed from the leaf chamber because of the small overpressure in the chamber (Li-Cor Inc. 2008), the observed leakage is purely the result of molecular diffusion through the gasket material.

The CO₂ leakage from a closed, empty chamber has been found to be constant, independently of the surrounding environmental conditions (empty chamber measurements conducted across the season 2011). There were no significant differences between measurements with an empty LAF and a LAF with test leaves (AL); consequently, the CO₂ leakage using LAF is only depending on the concentration gradient and not on wind speed around the cuvette or number and size of AF. The diffusion rates estimated from indoor measurements of an empty chamber sealed with two black gaskets and with AL inside the LAF did not change (0.38 mol s⁻¹, in both cases). This supports our first hypothesis (H1) that turbulence around the cuvette does not affect the gas exchange measurements in the absence of any LMP.

Measurements with AL without LAF demonstrated a different phenomenon, which can be explained with the theory described above. Correction of the CO₂ concentration inside the chamber with an empty chamber reference has been suggested by most manufacturers and described by, for example, Bernacchi *et al.* (2001). Attaching leaves inside the chamber will create small pores or air channels (LMP) between the gaskets and the sides of leaf veins or grass leaves,

as seen in Fig. 3c. Clearly, the number and sizes of the pores will vary between all individual leaves and thus the mass flow of air out of the chamber will vary from sample to sample and maybe even depend on the pressure applied when pressing the gaskets against each other (Flexas *et al.* 2007). The theory including the boundary layer around the gaskets explains why LMP lead to a decreased CO₂ leakage. It is the consequence of the reduced CO₂ concentration gradient across the gasket, which results from the dilution of the CO₂ concentration in the boundary layer (C_{bl}) on the outer side of the gasket with air from inside the chamber (C_i). The effectiveness of the reduction of the CO₂ concentration gradient depends on three parameters: (1) the concentration difference $C_a - C_i$; (2) the size and amount of LMP; and (3) the development and size of the boundary layer around the gasket (Fig. 2a). In Fig. 6, this phenomenon can be seen as a less steep slope when LMP are present. The fact that none of the two lines in Fig. 6 (AL inside and without LAF) intercept the x -axis (zero flux), but are higher, at ambient CO₂ concentrations, where no CO₂ concentration gradient should be present, supports the theory further. The concentrations directly at the outer surface of the gasket and the thickness of the boundary layer at the outside of the gasket determine the gradient. The consequence of the lower diffusion coefficient due to LMP is that the manufacturer's correction will overcorrect the diffusion rate across the gasket because it neglects the change in the CO₂ gradient across the gaskets. The estimated k -value from measurements with LMP are significantly lower than those given by the manufacturers (0.32 \pm 0.02 compared to 0.46 mol s⁻¹) and even though we used black gaskets as a reference, the diffusion rate k is still lower when LMP are present (0.32 \pm 0.02 to 0.38 \pm 0.02 mol s⁻¹).

Long & Bernacchi (2003) found that the CO₂ leakage varied depending on the type of leaf and suggested to use dead leaf material of the investigated species as reference for correction. In contrast, Flexas *et al.* (2007) showed in laboratory experiments that the rate of CO₂ leakage decreased

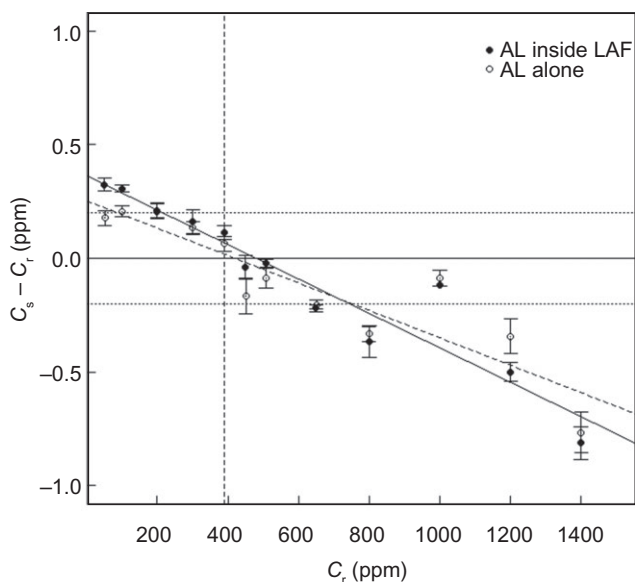


Figure 6. Effects of chamber reference CO₂ concentrations (C_i) on $C_s - C_i$, the difference between the sample and reference air CO₂ concentration, using artificial leaves (AL) either inside a leaf adaptor frame (LAF) (●) or without (○). The solid and the dashed lines are the linear regressions in the case where AL are attached inside a LAF or not, respectively. The two horizontal dotted lines represent the detection limits for the Li6400 (Li-Cor Inc. 2008) and the vertical dotted line is the CO₂ concentration in the surroundings during measurements.

when attaching a dead boiled leaf inside the chamber, explaining that most of the leakage from the chamber takes place in the interface between the gaskets because of different structures in leaf surface and not through the gaskets. Rodeghiero *et al.* (2007) concluded that the CO₂-diffusive molar flow rate ($\mu\text{mol CO}_2 \text{ s}^{-1}$) increased with LMP and thereby increased the CO₂ diffusion across the gasket, which supports Long & Bernacchi (2003) but disagrees with the interpretations of Flexas *et al.* (2007). Individually, these studies (Long & Bernacchi 2003; Flexas *et al.* 2007; Rodeghiero *et al.* 2007) support different parts of the theory of the present study.

The studies by Flexas *et al.* (2007) and Rodeghiero *et al.* (2007) conclude that a dead leaves correction strongly influenced the parameterization of the FvCB model and also agreed in their conclusion that correction of data using a constant diffusion coefficient are not useful. The two papers are both dealing with the hypothesis that minimizing the CO₂ concentration gradient can improve the accuracy of measurements and following photosynthetic parameterization. Testing the influence of enclosure of the leaf chamber in plastic bags both studies resulted in the reduction of CO₂ diffusion across the gaskets (Flexas *et al.* 2007) or the CO₂-diffusive molar mass flow (Rodeghiero *et al.* 2007). However, the studies do only conclude that a decrease in CO₂ concentration gradient is resulting in an improved parameterization, but no explanations of how this is related with LMP have been suggested.

All mentioned studies of the CO₂ leakage problem have been performed in laboratory environments where wind did not disturb the air surrounding the leaf chamber. According to our theory, laboratory experiments like the ones described above, can lead to misleading conclusions about the diffusion leakage.

Our result did not show any difference between empty LAF or LAF + AL measurements with and in windy or still air conditions at the outer gasket. However, in the absence of LAF, we found a significant influence of AL both under windy and not windy conditions. Under calm conditions, the LMP lead to a decrease in the rate of CO₂ leakage, which supports the findings of Flexas *et al.* (2007) and can be explained by the diluted outer surface CO₂ concentration and thus reduced gradient between C_i and C_{bl} . Results from bag experiments show a similar reduction of CO₂ leakage and can be explained by the same theory as described above. When Rodeghiero *et al.* (2007) found a reduction in the CO₂ leakage from LMP after enclosure of the chamber with a bag, it is due to a drop in the CO₂ concentration gradient between inside of the bag and C_i .

Under windy conditions, the boundary layer thickness is reduced, and therefore, the CO₂ gradient that controls the CO₂ diffusion will approach the difference between the CO₂ concentration inside and outside of the chamber. Thus, as suggested by Long & Bernacchi (2003), there will be an increased diffusion leakage. This is supported by our results.

Field conditions imply a varying disturbance of the boundary layer, and thus, a correction must take the thickness of the layer as depending on wind speed into account. This is virtually impossible because the wind speed close to the gasket is unknown and variable. We therefore advocate avoiding LMP and propose to seal irregular shoot and leaf structures with LAFs fitted to the actual leaf structure, such as the LAF developed in this study. This will lead to reproducible results that can be corrected with the methods proposed by the manufacturers. Our results support the correctness of these methods in the absence of LMP as will be discussed in the next section.

The use of LAF and the influence on respiration estimations

Gas exchange measurements on small leaves like grasses are challenging because of the small CO₂ fluxes that enhance the demand of accuracy, especially concerning respiration measurements. Our study has proven that LAF seal LMP in a way that only the diffusion across the gasket needs to be considered as a source of error. Li-Cor provides a flow-dependent diffusion rate k of 0.46 (Li-Cor Inc. 2008). Licor's k -value was found to be reproducible in our study, which has also been the case in earlier studies (e.g. Flexas *et al.* 2007; Rodeghiero *et al.* 2007). From this, the correction term provided by Li-Cor seems to be a good approach for correction of gas exchange data. Using LAF necessitates the use of two black gaskets since the white material is too sensitive to the shape folds of the LAF. We found different diffusion coefficients (k) using different gaskets or using LAF; which shows the importance

to choose an appropriate k -value for correction. We did not find any difference in k -values using LAF or only an empty chamber with two black gaskets. This is why we only presented the difference in correction with the LiCor provided k and our k_{BB} value.

The earlier mentioned correction using an empty chamber reference of a given CO_2 concentration inside the chamber has been suggested and used several times (e.g. Bernacchi *et al.* 2001; Albert *et al.* 2011b). Since no LMP are present when using LAF, this correction seems reasonable as long as the empty chamber measurements are performed under the same CO_2 regime as the measurements. Contrary to the study by, for example, Flexas *et al.* (2007), our parameterization of parameter estimates from the FvCB model only showed effects of the correction on the respiration value (R_{light}). Like the photosynthesis estimates from the FvCB model, results from the light response data only showed effects of the correction on the respiration parameter (R_{dark}), too. However, measured dark respiration (R_D) taken under ambient CO_2 concentrations was not changed by any of the correction methods. We conclude that such R_D measurements can serve as a true reference.

Unexpectedly, there was no difference between the influences of the two k -values (k and k_{BB}). The only difference between the correction methods on the parameterized estimates was between the R_{light} corrected with k_{BB} or ΔC_E ($P = 0.015$). A trend was also seen comparing R_{light} corrected with k and ΔC_E ($P = 0.15$). The R_{light} was estimated from the ACi curves where the C_r changed. Other parameters were obtained at stable C_r (1400 or 390 ppm), where the small changes in ΔC_E over C_r caused by the machine have a larger influence (clearly shown in, e.g. Fig. 5).

No significant differences between the three respiration estimates (R_{light} , R_{dark} , R_D) were found, when data was corrected with either of the k -values ($P > 0.35$) or ΔC_E ($P = 0.48$), proving that avoidance of LMP clearly improves leaf gas exchange measurements.

Beyond the problem of direct CO_2 leakage through LMP, a study by Pons & Welschen (2002) challenged the assumption that photosynthesis and respiration measured in the chamber are only related to leaf area between the inner boundaries of the gaskets, that is, the cuvette opening. Pons & Welschen (2002) argued that the leaf tissue between the gaskets is continuously contributing with a respiratory CO_2 flux transported through the gasket to the inside of the chamber. In addition here, LAF can be argued to improve the accuracy of measurement. Leaves inside LAF are sealed with blue tack, which eliminates the space around the leaf surface and the sealing material since the blue tack has been shown to be strongly gas tight. The only path for a small flux from the respiring leaf area will then be through the leaf tissue during measurements. The effect of lateral CO_2 diffusion inside leaves on the rate of photosynthesis has been shown to be very small and only over less than 0.3 mm (Morison & Lawson 2005). Thus, it can be neglected using LAF.

Several studies showed that leaf respiration rates are not sensitive to elevated CO_2 concentrations (e.g. Jahnke 2001;

Bruhn *et al.* 2002; Jahnke & Krewitt 2002). Any CO_2 response analysis requires proper correction of the CO_2 -diffusive leakage because this does also depend on the cuvette internal concentration via the concentration gradient compared to the ambient air concentration. If the correction overcorrects the diffusion, the corrected values might in fact falsely indicate even an increase of leaf respiration with increasing CO_2 concentration instead of a possibly expected but apparently non-existing product inhibition. The LAF technique can provide new insight by eliminating a major error regarding the accuracy of leaf gas exchange measurements of small fluxes in small leaf chambers. The advantage of the empty chamber correction is, however, that it also corrects for the so far unexplained machine-dependent systematic deviations that have been shown in Fig. 4 and can also be seen as systematic patterns of residuals in the other experiments (Figs 5 & 6). The origin of these systematic errors still remains to be investigated.

CONCLUSIONS

Certain leaf structures cause small holes or LMP across the contact zone of the upper and lower gaskets of gas exchange leaf cuvettes. Including the effects of such pores on the concentration in the boundary layer outside the cuvette and thereby reducing the concentration difference across the gasket, we were able to explain, at first glance, the counterintuitive reduction of CO_2 diffusion rates through the presence of LMP. The involvement of the boundary layer makes the effects of LMP on diffusion across the gasket wind speed dependent. Because the wind speed in field gas exchange measurements cannot be controlled, LMP need to be avoided. We showed that this can successfully be done with LAF, which we developed for this purpose. When avoiding LMP, the usual correction methods that describe diffusion through the gasket can be applied with large confidence. However, if possible, correction by means of empty chamber measurements done at same environmental conditions is the best correction resulting in most reliable results because it also corrects for measuring system-dependent biases that are unrelated to diffusion through the gaskets.

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REFERENCES

- Albert K.R., Ro-Poulsen H., Mikkelsen T.N., Michelsen A., Van der Linden L. & Beier C. (2011a) Interactive effects of elevated CO_2 , warming, and drought on photosynthesis of *Deschampsia flexuosa* in a temperate heath ecosystem. *Journal of Experimental Botany* **62**, 4253–4266.

- Albert K.R., Ro-Poulsen H., Mikkelsen T.N., Michelsen A., Van Der Linden L. & Beier C. (2011b) Effects of elevated CO₂, warming and drought episodes on plant carbon uptake in a temperate heath ecosystem are controlled by soil water status. *Plant, Cell & Environment* **34**, 1207–1222.
- Bernacchi C.J., Singsaas E.L., Pimentel C., Portis Jr, A.R. & Long S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell & Environment* **24**, 253–259.
- Bruhn D., Mikkelsen T.N. & Atkin O.K. (2002) Does the direct effect of atmospheric CO₂ concentration on leaf respiration vary with temperature? Responses in two species of *Plantago* that differ in relative growth rate. *Physiologia Plantarum* **114**, 57–64.
- Dubois J.-J.B., Fiscus E.L., Booker F.L., Flowers M.D. & Reid C.D. (2007) Optimizing the statistical estimation of the parameters of the Farquhar-von Caemmerer-Berry model of photosynthesis. *The New Phytologist* **176**, 402–414.
- Flexas J., Díaz-Espejo A., Berry J., Cifre J., Galmés J., Kaldenhoff R., Medrano H. & Ribas-Carbó M. (2007) Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. *Journal of Experimental Botany* **58**, 1533–1543.
- Jahnke S. (2001) Atmospheric CO₂ concentration does not directly affect leaf respiration in bean or poplar. *Plant, Cell & Environment* **24**, 1139–1151.
- Jahnke S. & Krewitt M. (2002) Atmospheric CO₂ concentration may directly affect leaf respiration measurement in tobacco, but not respiration itself. *Plant, Cell & Environment* **25**, 641–651.
- Lambers H., Chapin F.S. & Pons T.L. (1998) *Plant Physiological Ecology* (eds A.J. Shaw & B. Goffinet). Springer-Verlag New York Inc., New York, NY, USA.
- Li-Cor Inc. (2008) *Using the LI-6400/ LI-6400XT Portable Photosynthesis System Version 6*. Li-Cor Inc., Lincoln, NE, USA.
- Long S.P. & Bernacchi C.J. (2003) Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany* **54**, 2393–2401.
- Mikkelsen T.N., Beier C., Jonasson S., et al. (2008) Experimental design of multifactor climate change experiments with elevated CO₂, warming and drought: the CLIMATE project. *Functional Ecology* **22**, 185–195.
- Morison J. & Lawson T. (2005) Lateral diffusion of CO₂ in leaves is not sufficient to support photosynthesis. *Plant* **139**, 254–266.
- Nobel P.S. (1991) *Physicochemical and Environmental Plant Physiology*. Academic Press Inc., San Diego, CA, USA.
- Pons T.L. & Welschen R.A.M. (2002) Overestimation of respiration rates in commercially available clamp-on leaf chambers. Complications with measurement of net photosynthesis. *Plant, Cell & Environment* **25**, 1367–1372.
- Pons T.L., Flexas J., Von Caemmerer S., Evans J.R., Genty B., Ribas-Carbo M. & Brugnoli E. (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**, 2217–2234.
- R Development Core Team (2010) *R: A Language and Environment for Statistical Computing*. R Development Core Team, Vienna, Austria.
- Rodeghiero M., Niinemets Ü. & Cescatti A. (2007) Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: how erroneous are the estimates of Farquhar et al. model parameters? *Plant, Cell & Environment* **30**, 1006–1022.
- Smith W.K., Schoettle A.W. & Cui M. (1991) Importance of the method of leaf area measurement to the interpretation of gas exchange of complex shoots. *Tree Physiology* **8**, 121–127.

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